Yonago Acta medica 2000;43:19–25

Scanning Electron Microscopic Observations on the Intracellular Structures of the Ciliated Tracheal Epithelium—Especially on the Morphological Differences between Conventional Rats and Specific Pathogen-Free Rats

Miwako Hanamoto and Takao Inoué

Second Department of Anatomy, Faculty of Medicine, Tottori University, Yonago 683-0826, Japan

The three-dimensional architecture of the intracellular structures of the tracheal ciliated epithelium was studied by scanning electron microscopy, paying particular attention to the morphological difference between conventional rats and specific pathogen-free (SPF) rats. The surface of the trachea was densely covered with cilia in conventional rats, but less in SPF rats. The smooth endoplasmic reticulum (ER) formed a three-dimensional tubular meshwork under the basal bodies in both rats. In conventional rats, the Golgi apparatus was highly developed and many Golgi vesicles were attached to the lateral margin of the Golgi cisternae. In addition, the rough ER spread around the nucleus. In the SPF rats, however, the Golgi appatatus was not so highly developed and the rough ER was scarcely visible. The development of the Golgi apparatus and rough ER observed in the conventional rats indicate the active protein synthesis for the formation of the cilia which plays an important role in antimicrobial defense mechanisms.

Key words: ciliated epithelium; intracellular structure; scanning electron microscopy; specific pathogen-free rat; trachea

Although laboratory animals are frequently exposed to a variety of microorganisms, little attention has been paid in morphological studies to the conditions in which the animals were kept. The surface of the trachea is densely covered with ciliated cells which play an important role in eliminating foreign bodies by ciliary movement, when rats are kept under standard laboratory conditions (Alexander et al., 1975). In contrast, less ciliated cells are observed when rats are housed in germ free conditions (Jeffery and Reid, 1975). It is then easily speculated that the ultrastructures of ciliated cells are different between animals raised in conventional conditions and pathogen-free ones.

The smooth endoplasmic reticulum (ER), distributed all over the ciliated cells, shows enzymatic activity such as acethylcholinesterase (Graf and Stockinger, 1966), glucose-6-phosphatase (Kanamura, 1975; Hume and Burchell, 1996) and peroxidase (Kataoka, 1971; Watanabe, 1980; Christensen et al., 1981; Aoki et al., 1986; Sakai et al., 1989; Kinbara et al., 1992). In particular, acetylcholinesterase in the ER beneath the basal body is closely associated with the ciliary movement. Additionally, the endogenous peroxidase activity has not been localized in tracheal mucosal epithelial cells of specific pathogen-free (SPF) rats, but it became localized in the epithelial cells after natural infection with *Micoplasma pulmonis* (Kinbara et al., 1992).

In this study, we studied the three-dimensional architecture of the intracellular structures

Abbreviations: DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; SEM, scanning electron microscopy; SPF, specific pathogen-free; TEM, transmission electron microscopy

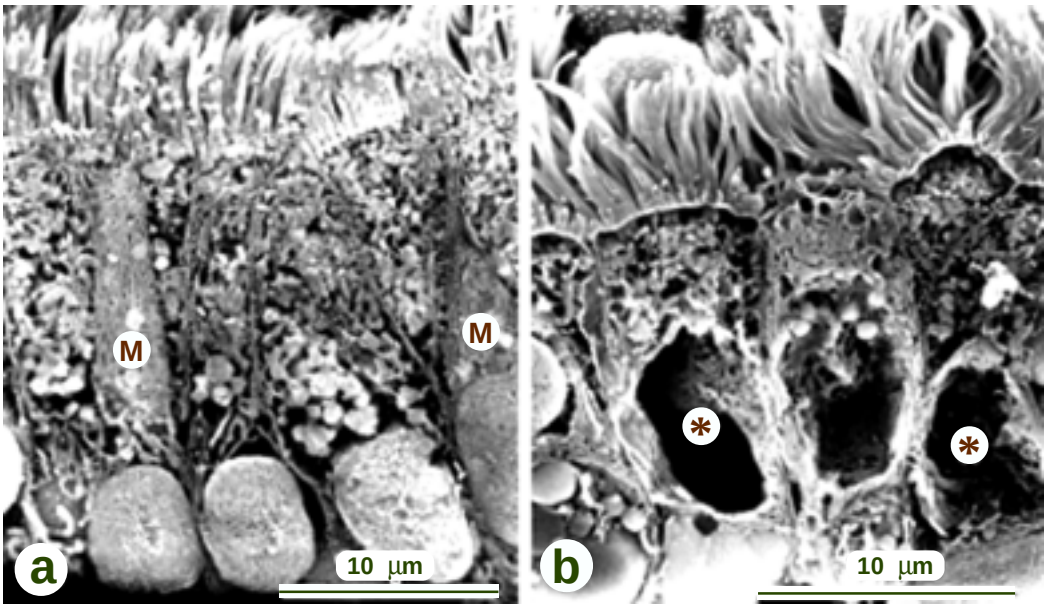

Fig. 1. Longitudinally fractured surfaces of the tracheal epithelial cells of a conventional rat (**a**) and a specific-pathogen free (SPF) rat (**b**). **a**: The surface of the epithelium is uniformly covered with cilia. Mucous cells (M) are often visible between the ciliated cells. × 2,400. **b**: Ciliated cells and non-ciliated cells are visible. The apical surface of the non-ciliated cells are covered with short microvilli. Mucous cells are sometimes seen. Asterisks show the perinuclear intracellular structures exposed by the removal of the nucleus during the specimen preparation procedure. × 2,800.

by scanning electron microscopy (SEM), paying particular attention to the morphological differences between conventional rats and SPF ones.

Materials and methods

Ten female adult Wistar rats conventionally raised on standard laboratory chow and water ad libitum were used as the control animals. Ten female adult Wistar SPF rats, purchased from Japan SLC, Ind. (Shizuoka, Japan), were used as the experimental animals. The SPF rats were selected from a carefully monitored stock declared free of the following specific pathogens: Sendai virus, Sialodacryoadenitis virus, Hanta virus, *Psedomonas aeruginosa, Salmonella* spp., *Pasteurella pneumotropica, Bordetella bronchiseptica, Streptococcus pneumoniae, Corynebacterium kutscheri, Mycoplasma* spp., Tyzzer's organism, *Syphacia* spp., *Giardia* spp., *Spironucleus* spp., *Trichomonas* spp. and *Entamoeba* spp.

SEM specimens were fundamentally prepared by the A-O-D-O method (Tanaka and Mitsushima, 1984). After the animals were anaesthetized with an intraperitoneal injection of Nembutal (50 mg/kg body weight), they were fixed by perfusion with a mixture of 0.5% glutaraldehyde and 0.5% formaldehyde (1/10 mol/L phosphate buffer, pH 7.4). The tracheas were then removed from the animals and excised into small pieces. After rinsing with the buffer, they were immersed in 25% and 50% dimethyl sulfoxide (DMSO) for 30 min each. The specimens were frozen on a metal plate chilled with liquid nitrogen and fractured into two pieces using a razor blade and a hammer. The fractured pieces were then placed in 50% DMSO solution and thawed at room temperature. After they were completely rinsed in the buffer, they were placed in 0.1% osmium tetroxide solution (1/10 mol/L phosphate buffer,

pH 7.4) and left standing for 3 days or more at 20˚C. After rinsing with the buffer, the specimens were postfixed in 1% osmium tetroxide for 1 to 2 h. They were then rinsed and treated with 1% tannic acid and 1% osmium tetroxide for conductive staining (Murakami, 1974). The specimens were dehydrated through a graded ethanol series, replaced with t-butyl alcohol and finally dried in a freeze-drier (ES-2020, Hitachi Ltd., Tokyo, Japan) (Inoué and Osatake, 1988). The dried specimens were coated with platinum of about 2 nm by an ion coater with a rotating stage (VX-10R, EIKO Engineering Co. Ltd., Mito, Japan) and observed with a field emission SEM (S-4500, Hitachi Ltd.) operated at 7 to 15 kV.

Results

The surface of the trachea was covered with three types of epithelial cells: ciliated, non-ciliated and mucous cells (Fig. 1).

Cytoplasmic matrices which prohibit the visualization of intracellular structures were completely removed by the osmic maceration procedure of the A-O-D-O method; consequently, both the outer and intracellular structures of ciliated cells could be demonstrated in three dimensions by SEM.

Common ultrastructural findings of both conventional and SPF rats

Basal bodies, extensions of the interior of the cilia, were lined beneath the apical plasma membrane (Fig. 2). The smooth ER formed a three-dimensional tubular meshwork under the basal bodies comprised of tubules of about 70 to 80 nm in diameter. The nucleus, located in the basal portion of the ciliated cells, was sometimes removed during the specimen preparation procedure, thus the perinuclear structures could be often observed. The Golgi apparatus, which was composed of vesicles, vacuoles and parallel-arranged cisternae, was located in the

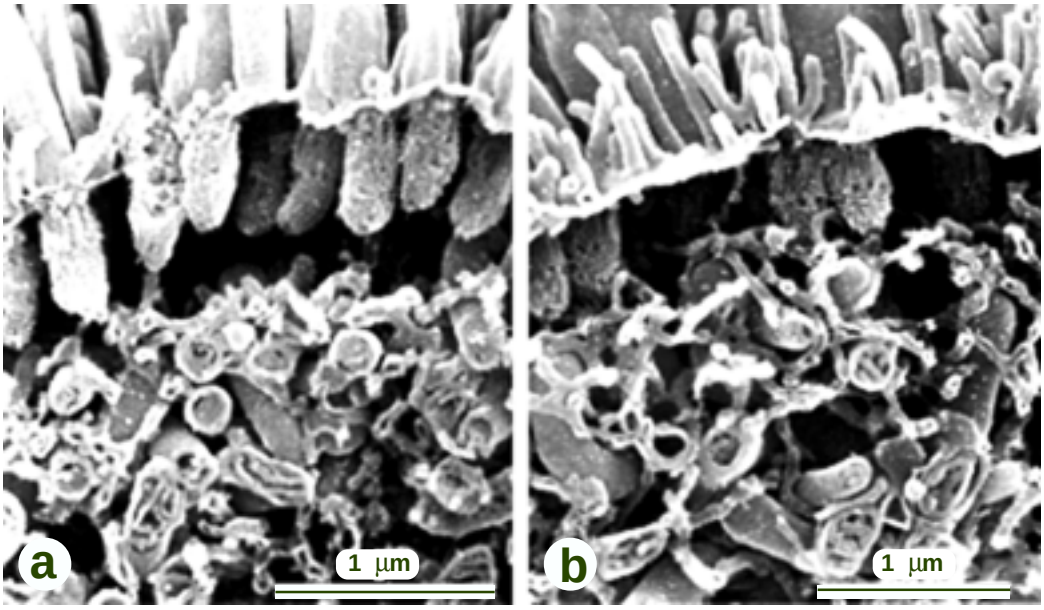

Fig. 2. Fractured surfaces of the apical portion of tracheal ciliated cells of a conventional rat (**a**) and SPF rat (**b**). Basal bodies are lined beneath the apical cell membrane. The tubular smooth endoplasmic reticulum forms a three-dimensional meshwork, partly trapping the mitochondria. Microvilli are more numerous in the SPF rat than in the conventional rat. **a**: × 36,000. **b**: × 26,000.

supranuclear region, usually representing a “U” shape as a whole (Fig.3). Most of the Golgi vacuoles and vesicles having been unconnected to membranous structures were generally removed during the specimen preparation procedure. However, Golgi vesicles firmly attached to the Golgi cisternae were preserved. The mitochondria, surrounded by the meshwork of the ER, were rod-shaped, branching and anastomosed to each other (Figs. 2 and 4).

The tubular smooth ER appeared to be continuous all over the interior of the ciliated cells. The rough ER with ribosomes was sometimes observed around the Golgi apparatus and nucleus.

Conventional rats

Ciliated cells were a major component of the epithelium. Mucous cells were often seen between the ciliated cells (Fig. 1a), and non-ciliated cells were scarcely visible.

Many cilia, together with a few microvilli, were distributed on the apical surface of the ciliated cells (Fig. 2a). The Golgi apparatus was highly developed and many Golgi vesicles were attached to the lateral margin of the Golgi cisternae (Fig. 3a). The Golgi stack was composed of five to eight flattened cisternae which were piled up in close parallel array.

The plate-like rough ER was observed around the nucleus (Fig. 4a). Many ribosomes were attached to the surface, some of them forming polysomes. The rough ER had a few fenestrations about 0.02 to 0.2 μm in diameter. The tubular smooth ER extended from the margin of the rough ER, linking to the adjacent rough ER.

SPF rats

The surface of the trachea was composed of ciliated cells and non-ciliated cells. The ciliated cells were less frequently observed in SPF rats than in conventional rats (Fig. 1b). Mucous cells were also less frequently visible than in conventional rats. The apical surface of the non-ciliated cells was covered with short micro-

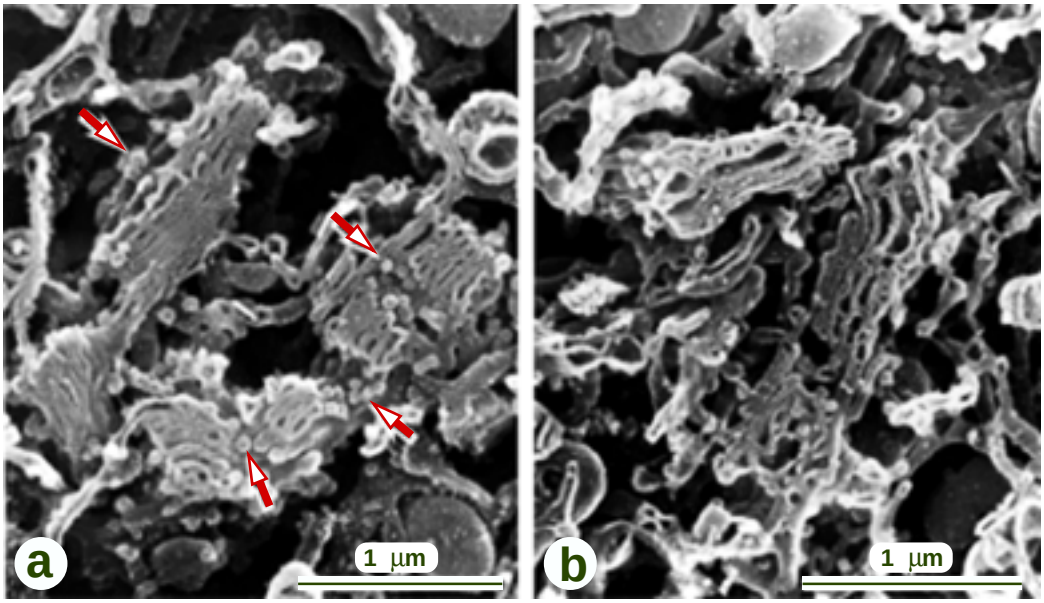

Fig. 3. The Golgi apparatus of tracheal ciliated cells in a conventional rat (**a**) and a SPF rat (**b**). **a:** The Golgi stack is composed of five to eight flattened cisternae which are piled up in a close parallel array. Note numerous Golgi vesicles (arrows). × 23,000. **b:** The Golgi stack is composed of four to seven fenestrated cisternae. × 28,000.

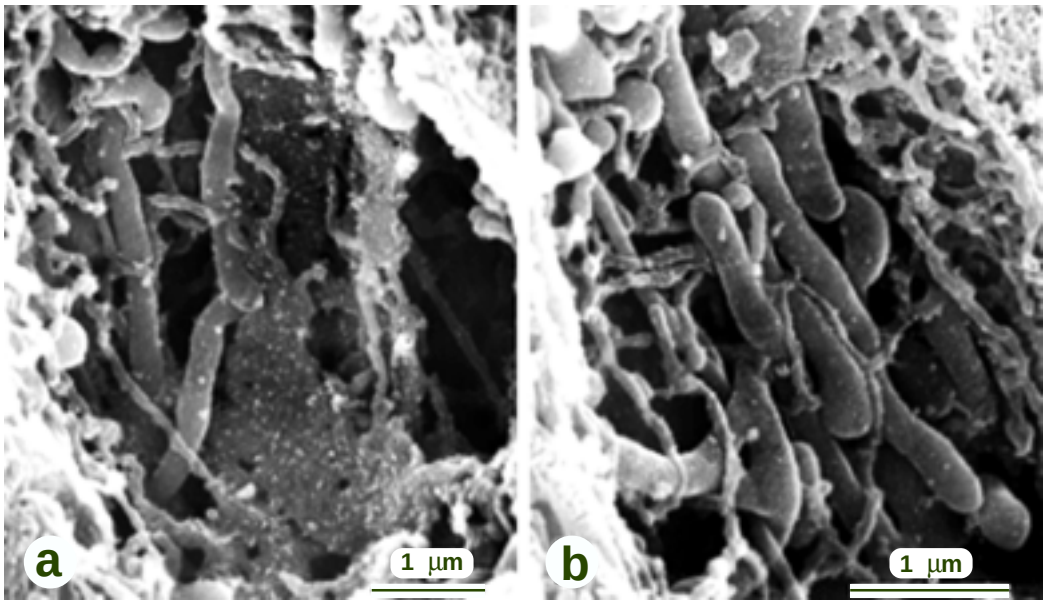

Fig. 4. The perinuclear intracellular structures exposed by the removal of the nucleus. **a**: A conventional rat. The tubular smooth endoplasmic reticulum extends from the margin of a plate-like rough endoplasmic reticulum. The rod-shaped mitochondria run parallel to the long axis of the cell. × 14,000. **b**: A SPF rat. A network of the tubular smooth endoplasmic reticulum is observed in the perinuclear region; the rough endoplasmic reticulum is not as highly developed as in conventional rats. × 17,000.

villi, bulging into the tracheal lumen. The cilia were not so highly developed as in conventional rats. Instead, numerous microvilli were visible (Fig. 2b). The Golgi apparatus was not so highly developed either. The Golgi stack was composed of four to seven cisternae. Golgi vesicles attached to the Golgi cisternae were fewer than those in conventional rats.

The ER in the perinuclear space was mostly smooth and tubular. The rough ER was scarcely visible (Fig. 4b).

Discussion

The intracellular structures of tracheal ciliated cells have been precisely examined by transmission electron microscopy (TEM) (Rhodin and Dalhamn, 1956; Jefferry and Reid, 1975; Marin et al., 1979). However, it has been difficult to understand the three-dimensional configuration of intracellular structures by two-dimensional TEM images. Although the development of specimen preparation for SEM made it possible to demonstrate intracellular structures three-dimensionally (Tanaka, 1980; Tanaka and Naguro, 1981; Inoué, 1982; Tanaka and Mitsushima, 1984; Inoué, 1985; Inoué and Osatake, 1989), intracellular structures of the tracheal ciliated epithelium have not been sufficiently studied by SEM. The osmic maceration procedure of the A-O-D-O method (Tanaka and Mitsusima, 1984) was effective to remove excess cytoplasmic matrices, thus intracellular structures such as the mitochondria, ER and Golgi apparatus were three-dimensionally observed as shown in Figs. 1 to 4.

The tracheal surface of conventional rats was densely covered with cilia as reported by Alexander et al. (1975). In contrast, there were fewer ciliated cells in SPF rats than in conventional rats. Non-ciliated cells of SPF rats were more often visible than those of conventional rats. The mucous cells were more frequently observed in conventional rats than in SPF rats. This apparently indicates that ciliated cells and

mucous cells are closely related to microorganisms in the air.

The most interesting finding obtained in this study was a three-dimensional network of the smooth ER. This network appeared to be continuous all over the interior of the ciliated cells. Such continuity of the ER has been shown three-dimensionally in rat spermatids by SEM (Inoué, 1982). An intensive network was demonstrated beneath the basal bodies, partly enclosing the underlying mitochondria (Fig.2). An amorphous layer, referred to as the hypobasal hyaline zone, was described between the basal bodies and underlying mitochondria by light microscopy (Hioki, 1942). Later, electron microscopy proved that the hypobasal hyaline zone contains the smooth ER (Graf and Stockinger, 1966), which is identical to the intensive ER network demonstrated in this study.

Kanamura (1975) considered that tracheal ciliated cells probably require a large amount of glucose-6-phosphate for ciliary movement. In addition, acetylcholine has been proven to have a close relationship with this movement (Kordik et al., 1952; Burn, 1954; Salathe and Bookman, 1995). Graf and Stockinger (1966) proved acetylcholinesterase activity in the ER of rat respiratory ciliated cells using histochemical techniques. Rhodin (1960) speculated that mitochondria under the basal bodies furnish the energy required for the ciliary beat. The close relationship between the smooth ER, basal bodies and mitochondria indicates that these intracellular organelles under the basal bodies are engaged in the supplementation of energy for ciliary movement.

This study showed that the Golgi apparatus and rough ER were highly developed in conventional rats (Fig. 3), but less so in SPF rats. The development of the Golgi apparatus can be estimated by the increased number of the Golgi cisternae and Golgi vesicles. Although no significant morphological differences were noted in the smooth ER under the basal bodies (Fig. 2), the plate-like rough ER was seen in the perinuclear region only in conventional rats (Fig. 4a). Since proteins, including enzymes, are synthesized and processed in the Golgi apparatus and rough ER, it is reasonable that such intracellular structures are highly developed for ciliary formation and movement. Probably, protein synthesis can be activated in conventional rats, thus the tubular smooth ER transformed into a plate-like rough ER.

Aoki et al. (1986) demonstrated that peroxidase activity in the rat tracheal epithelium was higher in conventional rats than in SPF rats. Kinbara et al. (1992) also showed similar findings in peroxidase activity, suggesting that peroxidase plays a role in mucosal antimicrobial defense mechanisms. However, the relationship between the peroxidase activity and intracellular morphology has not been clarified. The present morphological study on intracellular structures has been proven useful as an another approach when considering the defense mechanism of tracheal ciliated cells.

Acknowledgments: The authors are grateful to Prof. H. Ikoma from the Dept. of Otorhinolaryngology and Prof. E. Shimizu from the Third Dept. of Internal Medicine, Faculty of Medicine, Tottori University, for their helpful discussions. The authors also express sincere thanks to Mr. H. Osatake and Mr. S. Morino for their technical assistance.

References

1 Alexander I, Ritchie BC, Maloney JE, Hunter CR. Epithelial surfaces of the trachea and principal bronchi in the rat. Thorax 1975;30:171–177.

2 Aoki M, Hirai K, Itoh M, Ogawa K. Peroxidase activity in rat tracheal epithelium and gland. Exp Mol Pathol 1986;44:83–92.

3 Burn J. Acetylcholine as a local hormone for ciliary movement and the heart. Pharmacol Rev 1954;6:107–112.

4 Christensen TG, Blanchard GC, Nolley G, Hayes JA. Ultrastructural localization of endogenous peroxidase in the lower respiratory tract of the guinea pig. Cell Tissue Res 1981;214:407–415.

5 Graf J, Stockinger L. Endoplasmatisches Retikulum und Reizleitung im Flimmerepithel. Z Zellforsch 1966;72:184–192.

6 Hioki K. Zytologische Untersuchungen über das Flimmerepithel der menschlichen Trachea mit besonderer Berücksichtigung der Beziehung zwischen Flimmer- und Becherzellen. Cytologia

1942;12:326–346.
7 Hume R, Burchell A. The glucose-6-phosphatase enzyme in developing human trachea and oesophagus. Histochemi J 1996;28:141–147.
8 Inoué T. The continuity of endoplasmic reticulum in rat spermatids observed by scanning electron microscopy. J Electron Microsc 1982; 31:261–263.
9 Inoué T. High resolution SEM cytology. In: Müller M, Becker RP, Boyde A, Wolosewick JJ, eds. Science of biological specimen preparation. Chicago: SEM Inc.; 1985. p. 245–256.
10 Inoué T, Osatake H. A new drying method of biological specimens for scanning electron microscopy: the t-Butyl alcohol freeze-drying method. Arch Histol Cytol 1988;51:53–59.
11 Inoué T, Osatake H. Three-demensional demonstration of the intracellular structures of mouse mesothelial cells by scanning electron microscopy. J Submicrosc Cytol Pathol 1989; 21:215–227.
12 Jeffery PK, Reid L. New observations of rat airway epithelium: a quantitative and electron microscopic study. J Anat 1975;120:295–320.
13 Kanamura S. Ultrastructural localization of glucose 6-phosphatase activity in tracheal epithelium of the rat. J Anat 1975;119:499–504.
14 Kataoka K. Fine structural localization of peroxidase activity in the epithelium and the gland of the rat larynx. Histochemie 1971;26: 319–326.
15 Kinbara M, Ueda T, Hirai K. Expression of peroxidase activity in rat tracheal epithelial cells associated with Mycoplasma pulmonis. Am J Physiol 1992;262:L92–L99.
16 Kordik P, Bülbring E, Burn JH. Ciliary movement and acetylcholine. Br J Pharmacol 1952;7: 67–79.
17 Marin ML, Lane BP, Gordon RE, Drummond E. Ultrastructure of rat tracheal epithelium. Lung 1979;156:223–236.
18 Murakami T. A revised tannin-osmium method for non-coated scanning electron microscope specimens. Arch Histol Jpn 1974;36:189–193.
19 Rhodin J. Ultrastructure of the tracheal ciliated mucosa in rat and man. Ann Otol Rhinol Laryngol 1960;68:964–974.
20 Rhodin J, Dalhamn T. Electron microscopy of the tracheal ciliated mucosa in rat. Z Zellforsch 1956;44:345–412.
21 Sakai J, Ueda T, Hirai K. Postnatal development of peroxidase activity in the laryngeal and tracheal epithelia and glands of specific pathogen free rats. Acta Histochem Cytochem 1989;22: 421–430.
22 Salathe M, Bookman RJ. Coupling of $[Ca^{2+}]i$ and ciliary beating in cultured tracheal epithelial cells. J Cell Sci 1995;108:431–440.
23 Tanaka K. Scanning electron microscopy of intracellular structures. Int Rev Cytol 1980;68: 97–125.
24 Tanaka K, Mitsushima A. A preparation method for observing intracellular structures by scanning electron microscopy. J Microsc 1984;133(Pt 2):213–222.
25 Tanaka K, Naguro T. High resolution scanning electron microscopy of cell organelles by a new specimen preparation method. Biomed Res 1981;2 Suppl:63–70.
26 Watanabe K. Localization of peroxidase activity in tracheal epithelium. Ann Otol 1980;89:241–248.

(Received December 20, 1999, Accepted January 4, 2000)